Video Article

Probing for Mitochondrial Complex Activity in Human Embryonic Stem Cells

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Abstract

Mitochondria are cytoplasmic organelles that have a primary role in cellular metabolism and homeostasis, regulation of the cell signaling network, and programmed cell death. Mitochondria produce ATP, regulate the cytoplasmic redox state and Ca2+ balance, catabolize fatty acids, synthesize heme, nucleotides, steroid hormones, amino acids, and help assemble iron-sulfur clusters in proteins. Mitochondria also have an essential role in heat production. Mutations of the mitochondrial genome cause several types of human disorder. The accumulation of mtDNA mutations correlates with aging and is suspected to have an important role in the development of cancer. Due to their vitally important role in all cell types, the function of mitochondria must also be critical for stem cells. Key advances have been made in our understanding of stem cell viability, proliferation, and differentiation capacity. But the functional activity of stem cells, in particular their energy status, was not yet been studied in detail. Almost nothing is known about the mitochondrial properties of human embryonic stem cells (hESCs) and their differentiated precursor progeny. One way to understand and evaluate the role of mitochondria in hESC function and developmental potential is to directly measure the activity of mitochondrial respiratory complexes. Here, we describe high resolution clear native gel electrophoresis and subsequent in gel activity visualization as a method for analyzing the five respiratory chain complexes of hESCs.

Video Link

The video component of this article can be found at https://www.jove.com/video/724/

Protocol

High Resolution Clear Native Gel Electophoresis (hrCNE) for Mitochondrial Complex In-Gel Activity Assays

Human embryonic stem cells (hESCs) were maintained on a monolayer of ɣ-irradiated or mitomycin C-treated mouse embryonic fibroblasts (MEFs), then switched to growth on Matrigel for 5 days in MEF-conditioned medium using standard conditions. To remove contaminating MEFs, hESCs were re-plated on Matrigel and grown in MEF-conditioned medium for an additional 3 days. Immunofluorescence microscopy for pluripotency markers Oct-4 and SSEA-4 was used to confirm the lack of hESC differentiation.

1. Wash cells with warm 1x PBS, pH 7.4, followed by trypsinization (1ml of 1x trypsin per well in a 6-well plate) for 5 min.
2. Add an equal volume of fresh Trypsin Inhibitor (1mg/ml in 1x PBS, pH 7.4). Harvest cells and centrifuge at 200g, 5 min, room temperature.
3. Discard the supernatant and resuspend pelleted cells in 0.8 - 1.7ml (based upon the size of the cell pellet) of ice cold mitochondria isolation buffer (83mM sucrose; 6.6mM imidazole/HCl, pH 7.0) with freshly prepared 1´ Protease Inhibitor Cocktail. Incubate on ice for 10 min.
4. Dounce cells with a homogenizer (~40 - 50 strokes). Stain an aliquot of the dounced cells with trypan blue to check for completeness of homogenization using a light microscope.
5. Centrifuge at 20,000g for 10 min at 4°C to obtain a crude mitochondrial pellet that also contains nuclei and larger cell fragments. Decant and discard the supernatant and weigh the precipitated crude mitochondrial pellet. The pellet can be stored at -80 °C following instant freezing in liquid nitrogen if this is a stopping point.
   NOTE: Differential centrifugation or other gradient-based procedures can be used for isolating and collecting a pure mitochondrial membrane fraction. See ref. 4 for details. This alternative approach will yield the mitochondrial complex activity per mg of mitochondrial protein instead of complex activity per mg of cell weight or cell number. The concentration of mitochondrial proteins will need to be determined after purification to make this comparison. Also, a different isolation buffer may be required for this approach. For solubilization of the pure mitochondrial membrane fraction, see the note following step 6 below.
6. Solubilize the crude mitochondrial pellet on ice by vortexing with ice cold solubilization buffer (50mM NaCl, 50mM imidazole/HCl pH 7.0, 1mM EDTA, 2mM 6-aminohexanoic acid). 6-aminohexanoic acid can be replaced with the 1x Protease Inhibitor Cocktail. Use 35µl of solubilization buffer per 20mg of pellet weight. Add 20µl of 10% w/v digitonin per 20mg of pellet weight and mix by flicking the tube. Incubate for 5-10 min on ice. Dodecyl-b-D-maltoside or triton X-100 can be substituted for digitonin to solubilize the proteins. The choice of detergent and its quantity may affect the formation of supercomplexes or multimeric forms of mitochondrial complexes. For more information, see ref.1.

NOTE: The quantity of detergent required to solubilize a pure mitochondrial membrane fraction corresponds to a detergent/protein weight ratio ranging from 2-6g/g. The optimal ratio to solubilize mitochondrial membranes is 2.5g/g for dodecyl-b-D-maltoside, 3g/g for triton X-100, and 6g/g for digitonin.

7. Centrifuge the solubilized pellet at 100,000g for 15 min at 4°C. Collect the clear supernatant.
8. Add loading buffer to a final 5% w/v glycerol and 0.01% w/v Ponceau S (use a 50% w/v glycerol, 0.1% w/v Ponceau S loading buffer stock solution).
9. Load gel wells with a volume that contains 20-40mg of the crude mitochondrial pellet per lane. Use an acrylamide gradient gel (4 -13%) with a 3.5% stacking gel.
   NOTE: Gel preparation and electrophoresis conditions:
   For gel preparation, use an acrylamide/bis-acrylamide (AA/bisAA) 3 mix as follows: 48g of acrylamide and 1.5g of bisacrylamide (49.5% T, 3% C. Where T is total concentration of AA and bisAA and C is the percentage of crosslinker (bisAA) to total monomer).
   Gel buffer (3´): 1.5M 6-aminohexanoic acid; 75mM imidazole, pH 7.0

Gradient gel:	4%	13%	Stacking gel:	3.5%
AA/bisAA 3 mix	2 ml	6.5 ml		0.6 ml
Gel buffer 3´	8.3 ml	8.3 ml		2.7 ml
Glycerol		5 g		
H_2O	14.7 ml	5 ml		4.7 ml
Total volume	25 ml	25 ml		8 ml
10% APS	139 µl	125 µl		67 µl
TEMED	14 µl	12.5 µl		6.7 µl

Anode buffer: 25mM imidazole/HCl, pH 7.0
Cathode buffer: 50mM Tricine, 7.5mM imidazole/HCl, 0.02% w/v dodecyl-b-D-maltoside, 0.05% w/v deoxycholate, pH 7.0
Pour and run gels in a cold room (4-7 °C). The initial voltage used is 100V. When the sample has entered the stacking gel, the voltage is raised to 500V with the current limited to 15mA (for 0.15 ´ 14 ´ 14-cm gels). Electrophoresis is stopped after 3-4 h when the sharp line of the red Ponceau S dye approaches the gel front. Alternatively, a gel can be run overnight with constant voltage at 70-100V.

1. In-gel activity assays. The time of incubation for each assay depends on the efficiency of protein solubilization and the amount of material loaded per well. Complexes I, IV and V usually yield stronger signals than complexes II and III.

Complex I (CI)

Incubate the gel slice with CI buffer (5mM Tris-HCl, pH 7.4; 0.1mg/ml NADH; 2.5mg/ml NBT) at room temperature for several hours.

CI buffer: Add per 100ml of 5mM Tris-HCl, pH 7.4:

0.01g NADH

0.25g NBT

Fix the gel in 50% methanol and 10% acetic acid for 15-45 min. The fixed gel is preserved in 10% acetic acid.

Complex II (CII)

Incubate the gel slice with CII buffer (20mM sodium succinate; 0.2mM phenazine methasulfate; 2.5mg/ml NBT; 5mM Tris-HCl pH 7.4) at room temperature for several hours.

CII buffer: Add per 100ml of 5mM Tris-HCl, pH 7.4:

200µl 1M sodium succinate

80µl phenazine methasulfate (250mM PMS dissolved in DMSO)

0.25g NBT

Fix the gel as described for Complex I

Complex III (CIII)

Incubate the gel slice with CIII buffer (50mM sodium phosphate, pH 7.2; 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB)) at room temperature for several hours.

CIII buffer: Add per 100ml of 50mM sodium-phosphate, pH 7.2:

50mg diaminobenzidine tetrahydrochloride

Fix the gel as described for Complex I

Complex IV (CIV)

Incubate the gel slice with CIV buffer (50mM sodium phosphate, pH 7.2; 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB), 50µM horse heart cytochrome c) at room temperature for several hours.

CIV buffer: Add per 100ml of 50mM sodium-phosphate, pH 7.2:

50mg diaminobenzidine tetrahydrochloride

0.1g horse heart cytochrome c

Fix the gel as described for Complex I

Complex V (CV)

Incubate the gel slice with CV buffer (35mM Tris-base, 270mM glycine, pH 8.3 at 25°C, 14mM $MgSO_4$, 0.2% w/v $Pb(NO_3)_2$, 8mM ATP) at room temperature for several hours.

CV buffer: Per 100ml of solution add:

Tris-base	0.424g
Glycine	2.026g
$MgSO_4$	0.345g
$Pb(NO_3)_2$	0.29g

No need to adjust pH (should be 8.3) - do not use acid or base. Filter and add

ATP	0.441g

Fix the gel in 50% methanol for 30 min and transfer to water.

Discussion

In this video, we have described the extraction of mitochondrial protein complexes from human embryonic stem cells, their separation by high resolution clear native gel electrophoresis (hrCNE), and the subsequent analysis by in-gel catalytic activity assays. hrCNE resolves mitochondrial membrane protein complexes at a resolution comparable to that of blue native gel electrophoresis and is superior for in-gel activity assays. This technique can be employed not only to quantify mitochondrial respiratory complexes I-V but also to analyze the subunit composition of any mitochondrial membrane protein complex both in hESCs and in their derivative progeny.

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